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## Document heading

## Effect of simvastatin on paraoxonase 1 (PON1) activity and oxidative stress

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## ABSTRACT

**Objective:** To investigate the effect of simvastatin treatment on lipid profile and oxidative stress in hypercholesterolaemic Indian population and determine the effect of simvastatin treatment on the activity of paraoxonase (PON). **Methods:** Analyzed initially before medication administration and four months later after medication. Lipid and lipoprotein measurement were done by enzymatic kits, high density lipoprotein (HDL) was determined by phosphotungstic acid precipitation method and low density lipoprotein (LDL) was calculated by Friedewald's formula. Lipid peroxidation was measured by three markers namely, conjugated diene, total peroxide, and malondialdehyde. Conjugated diene was assayed by Buege and Aust method. Total peroxide was determined by FOX2 method. Malondialdehyde determination was carried out by Flemming method and total antioxidant status was determined by Ozacan. Paraoxonase activity was determined by measuring the absorbance increase of p-nitrophenol at 405 nm. Arylesterase activity was calculated from the molar coefficient of  $1.310 \text{ M}^{-1} \text{ cm}^{-1}$ . **Results:** Simvastatin significantly reduced total cholesterol, triglycerides, LDL, conjugated diene, total peroxide and MDA levels, where as antioxidant status was significantly increased. Besides, simvastatin significantly increased PON1 activity towards paraoxon. **Conclusions:** The results from the current study indicate simvastatin may have important antioxidant properties via increasing PON activity.

## 1. Introduction

Atherosclerosis is the primary cause of cardiovascular disease (CVD) and coronary heart disease (CHD). It is a leading cause of global morbidity and mortality in the modern world<sup>[1]</sup>. Atherosclerosis is a complex multi-factorial disease. The oxidative modification theory for atherosclerosis postulates that oxidation of low density lipoprotein (LDL) is the key factor for this pathogenesis<sup>[2]</sup>.

Oxidised LDL acts a chemo attractant for monocyte, transforms macrophage into foam cells. It exerts cytotoxic effects on endothelial cells, increases thrombocyte activation, and stimulates migration and proliferations of smooth muscles cells that lead to formations of atheromatous lesions<sup>[2,3]</sup>. Several potentially anti-atherogenic mechanisms have been associated with high density lipoprotein (HDL). These include both the protection of LDL against oxidation and attenuation of the biological activity of Ox-LDL<sup>[4–6]</sup>. Antioxidant and anti-atherogenic properties of a HDL are

noticed from associated enzyme, paraoxonase1 (PON1). Numerous studies have indicated that PON1 is largely responsible for HDL's anti-oxidative property<sup>[7–9]</sup>.

Moreover recent findings of two new PON members, PON2 and PON3 also showed the protective role to oxidative stress in tissues and plasma<sup>[10,11]</sup>. PON1 is a calcium dependent ester hydrolase which is primarily synthesized in liver and associated with HDL in the plasma<sup>[12–14]</sup>. PON1 inhibits LDL and HDL from oxidative modification and also scavenges oxidized phospholipids from LDL<sup>[7,8,15]</sup>. Moreover, various clinical epidemiological studies demonstrated that PON1 enzyme activity was inversely related to the risk of CHD and CVD<sup>[16–19]</sup>.

On the basis of clinical trial evidence, the most commonly prescribed lipid modifying therapies are the hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, more commonly known as statin. Competitive inhibition of this enzyme by the statin decreases hepatocyte cholesterol synthesis<sup>[20]</sup>. Statin also shows beneficial effect on other lipid parameters, including increases in HDL and decreases in triglycerides (TG). In general, statin is regarded as a remarkably safe and well tolerated class of drugs. Seven statins are now approved for clinical uses,

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which are simvastatin, cerivastatin, fluvastatin, lovastatin, pravastatin, atorvastatin and pitavastatin[20]. Though they all have a common function, but differ in term of chemical structures, pharmacokinetic profiles, and lipid modifying efficacy[21]. Simvastatin decreases serum cholesterol by reducing the production and increasing its rate of removal from the body [22]. It also reduces TC, LDL and TG levels and increases HDL levels. Simvastatin acts as an antioxidants, either directly or indirectly and may have beneficial effect on atherosclerosis apart from their effect on serum TC, it also involve in modifying endothelial functions.

The present study was to determine the effect of simvastatin treatment on lipid profile, oxidative stress and activity of PON in hypercholesterolaemic Indian population.

## 2. Materials and methods

Fifty two hypercholesterolaemic volunteers with 30–49 yr were selected for the study. Patients with diabetes, hypertension, hyperthyroidism, liver disease, renal dysfunction, history of myocardial infarction, coronary heart disease, cardiovascular disease, alcoholic, and smokers were excluded. Pregnancy, lactating women and participants taking hypolipidemic drugs and antioxidants vitamins were also excluded.

All subjects were undergone medical check-up at the Department of Cardiology, and were screened for medical history. A pre-tested questionnaire was used to collect information regarding medical history, smoking, and alcohol consumption from the participants. All cases with TC >240 mg/dL and LDL >170 mg/dL were administrated with simvastatin 10mg /day for 4 months. The outcomes were analyzed at 0 and 4 months after medication. After 4 months of simvastatin treatment, hypercholesterolemia subjects had physically examination. The anthropometric measurements included weight, height, waist and hip circumference. Blood pressure was also measured. Clinical laboratory investigations included a complete blood cell count, blood urea nitrogen, creatinine, fasting blood sugar, liver function tests [alanine aminotransaminase (ALT), aspartate amino transaminase (AST)], and lipid profile analysis.

All volunteers were properly instructed to fast for 12 hours before venipuncture. Venous blood was collected two times, before and after 4 months of simvastatin administration. 5 mL blood was collected in a plain tube without anticoagulant and was allowed to clot at room temperature then centrifuged at 3500×g for 10 mins. The parameters included serum TC, TG, HDL, LDL, conjugated diene (CD), malondialdehyde (MDA), total antioxidant status (TAS), oxidative stress index (OSI) and paraoxonase activity.

PON1 activities were determined by using paraoxon and phenyl acetate as substrates[23,24].

### 2.1. Paraoxon assay

Paraoxonase activity was determined by measuring the absorbance increase of p-nitrophenol at 405 nm. Adding 20  $\mu$  L of serum to 1 mL Tris buffer (100 mM, pH 8.0) containing 2 mM  $\text{CaCl}_2$  and 1.1 mM paraoxon. The rate of hydrolysis of paraoxon was followed by measuring the liberation of p-nitrophenol at 405 nm at 37 °C over 50 sec after 1 min lag time with spectrophotometer.

$$\text{Activity} = \frac{(\text{OD/min}) \times \text{assay volume}}{\epsilon \times \text{sample volume} \times \text{path length}}$$

The enzyme activity was calculated from the molar extinction coefficient ( $\epsilon$ ) of 18 700  $\text{M}^{-1} \text{cm}^{-1}$  at 405 nm, pH 8.0. Activity was expressed as catalytic concentration (U/L) which corresponds to the product of 1  $\mu$  M of p-nitrophenol/min/L of serum.

### 2.2. Arylesterase activity assay

The reaction mixture contained 1.0 mM phenylacetate and 0.9 mM calcium chloride in 10 mM Tris-HCl buffer at pH 8.0. The reaction was initiated by adding 20  $\mu$  L serum which was pre diluted at 1:20 ratio with 10 mM Tris-buffer at pH 7.4. Absorbance increase of phenol was measured at 270 nm and 37 °C using spectrophotometer.

$$\text{Activity} = \frac{(\text{OD/min}) \times \text{assay volume}}{\epsilon \times \text{sample volume} \times \text{path length}}$$

The enzyme activity was calculated from the molar coefficient of 1 310  $\text{M}^{-1} \text{cm}^{-1}$ . Activity was expressed as catalytic concentration (U/L) which corresponds to the product of 1 mM of phenol per minute per liter of serum.

The study was ethically cleared from the Institutional Ethical Committee. All participants signed the informed consent form before being enrolled for the study.

## 3. Results

Forty-nine of 52 patients (94.23%) completed the study. The withdrawal rate in this study was 5.76% (3/52). Simvastatin treatment was well tolerated and none of the patients experienced serious adverse effects in this study. Three patients discontinued treatment due to loss of follow up.

### 3.1. Demographic characteristics

The demographic characteristics of hypercholesterolaemic subjects were presented in Table 1. The mean age was 57.7 years. The study population had a greater proportion of female than male. Out of 52 patients, males were 18 (36.4%) and females were 34 (63.6%). The mean of body mass index (BMI), waist-hip ratio (WHR), systolic blood pressure (SBP), diastolic blood pressure (DBP), fasting blood sugar (FBS) of baseline were  $(25.0 \pm 3.7) \text{ kg/m}^2$ ,  $0.9 \pm 0.1$ ,  $(132.0 \pm 17.8) \text{ mmHg}$ ,  $(80.0 \pm 11.9) \text{ mmHg}$ , and  $(103.0 \pm 9.0) \text{ mg/dL}$ , respectively. As shown in Table 1, there were no significant changes in demographic characteristics after 4 months of simvastatin treatment.

### 3.2. Effect of simvastatin on lipid, CD, total peroxide, MDA, TAS, and OSI levels

The mean levels of total cholesterol (TC), TG, HDL, and LDL at baseline were  $(254.00 \pm 40.00) \text{ mg/dL}$ ,  $(185.00 \pm 20.00) \text{ mg/dL}$ ,  $(50.10 \pm 12.50) \text{ mg/dL}$  and  $(158.00 \pm 32.00) \text{ mg/dL}$ , respectively. The mean levels of CD, total peroxide, MDA, TAS, trolox equivalent/L and OSI at baseline were  $0.38 \pm 0.06$ ,  $[18.40 (13.60-26.60)] \mu \text{mol H}_2\text{O}_2/\text{L}$ ,  $(17.60 \pm 2.70) \text{ mmol/L}$ ,  $(0.81 \pm 0.14) \text{ mmol/L}$  and

2.42±0.73, respectively.

**Table 1**

Demographic characteristic of study subjects from baseline to four months ( $n=50$ ).

Parameters	Hypercholesterolaemic subjects ( $n=52$ )	
	Baseline	4 months
Age (years)	57.7±7.4	58.0±7.4
BMI (kg/m <sup>2</sup> )	25.0±3.7	26.6±3.4
Waist–hip ratio	0.9±0.1	0.9±0.9
SBP (mmHg)	132.0±17.8	132.0±25.4
DBP (mmHg)	80.0±11.9	74.0±11.8
FBS (mg/dL)	103.0±9.0	108.0±10.0

As shown in Table 2, Simvastatin treatment significantly decreased TC, TG, LDL, CD, total peroxide, MDA, and OSI levels by 24.5%, 24.4%, 22.4%, 4.4%, 13.0%, 15.2% and 24.0%, respectively.

Meanwhile, the mean TAS level significantly increased by 27.3%, however, there was no significant difference in level of HDL at 4 months treatment of simvastatin [(50.40±9.20) mg/dL] when compared with the baseline level [(50.1±12.5) mg/dL].

The percent (%) change of LDL was positively correlated with % change of TC ( $R^2=0.63$ ,  $P<0.05$ ). There was no

correlation between % change of TAS and % change of LDL ( $R^2=0.15$ ,  $P>0.05$ ). HDL level did not increase significantly by Simvastatin treatment and the % change of HDL level was not correlated with change of lipid peroxidation parameter ( $R^2=0.05$ ).

### 3.3. Effect of simvastatin on PON1 activity

The mean levels of PON1 activities towards paraoxon and phenyl acetate were (178±77)  $\mu$  mol/min/mL and (142±25) mmol/min/mL, respectively.

As shown in Table 3 and Table 4, there was significant increase in PON1 activity towards paraoxon (+13.4%,  $P<0.05$ ) but PON1 activity towards phenylacetate did not change significantly (+1.35%,  $P>0.05$ ) after 4 month of simvastatin treatment. However, PON1 activity toward phenylacetate increased significantly in male ( $P<0.05$ ). In this study, it was found non-significant difference of baseline PON1 activity towards paraoxon between male and female, but PON1 activity towards phenyl acetate was significantly different between male and female ( $P<0.05$ ).

The percent change of PON1 activity towards paraoxon was positively correlated with % change of TAS ( $R^2=0.30$ ,  $P<0.05$ ).

**Table 2**

Effect on serum lipid parameters, serum CD, total peroxide, MDA, TAS, and OSI levels ( $n=52$ ).

Parameters		Hypercholesterolaemic subjects	
		Baseline	4 months
Serum lipid parameters	TC (mg/dL)	254.00±40.00	199.00±36.00*
	TG (mg/dL)	185.00±20.00	141.00±15.00*
	HDL (mg/dL)	50.10±12.50	50.40±9.20
	LDL (mg/dL)	158.00±32.00	118.00±27.00*
Lipid peroxidation	CD	0.38±0.06	0.34±0.05*
	Total peroxide ( $\mu$ mol H <sub>2</sub> O <sub>2</sub> /L)	18.40 (13.60–26.60)	15.90 (11.10–24.00) *
	MDA (mmol/L)	17.60±2.70	14.80±2.90*
	TAS (mmol Trolox Equiv/L)	0.81±0.14	1.01±0.17*
	OSI	2.42±0.73	1.79±0.56*

\*: $P<0.05$ .

**Table 3**

PON1 activity at baseline and 4 month after simvastatin treatment ( $n=52$ ).

PON activity	Hypercholesterolaemic subjects	
	Baseline	3 months
PON 1 levels Paraoxon hydrolysis ( $\mu$ mol/min/mL) % change	178±77	196±90
Phenyl acetate hydrolysis (mmol/min/mL) % change	142±25	145±33

#### 4. Discussion

Oxidative modification of LDL is believed to be the underlying cause in pathogenesis of atherosclerosis[2]. With this concept the prevention of atherosclerosis and its consequence could be limited by reducing the ox-LDL levels and oxidative stress. Simvastatin is used as a drug to reduce lipid in hypercholesterolaemic patients. Moreover, Liao and colleague reported that simvastatin exerts beneficial cardiovascular effect independent of their lipid lowering property[25], which might be due to its antioxidant properties[26]. In the current study, four months of simvastatin administration on hypercholesterolaemic subjects significantly reduced TC, TG, and LDL. The reduction of lipid parameters can be explained on the basis of inhibitory effect on hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase by simvastatin, the rate limiting step in de novo cholesterol synthesis. This pathway decreases hepatocytes cholesterol synthesis and subsequently clear the LDL from the circulation by LDL receptor of hepatocytes cell surface. The effect of simvastatin in lowering TC, TG, and LDL levels obtained in our study were similar to those described by elsewhere[27,28]. It has been reported that simvastatin also has beneficial effect of raising HDL levels by unknown mechanism(s)[29,30]. However, the present study did not observe any rise in increase of HDL levels by simvastatin as previously reported by others[27,28]. These discrepancies may be partly explained by variations in the participants of the study, such as the severity of dietary control, exercise, genetic effect, patient behavior, and disease state.

Simvastatin exerts beneficial cardiovascular effect independent of their lipid lowering properties[25] and it might be due to its antioxidant effect[26] which is believed to come from HDL-associated enzymes, PON1. There are considerable numbers of potential pharmacological effect of various lipid lowering drugs on PON activity. The increase in serum PON1 activity by statin is still unclear due to the disagreement of results from earlier studies. In this study, simvastatin significantly increased PON1 activity towards paraoxon ( $P<0.05$ ), however, there was a non-significant trend for the increase in the PON1 activity towards phenyl acetate ( $P>0.05$ ). Similar results were observed in simvastatin treatment that was studied by Tomas and colleague[31]. PON1 activity has been shown to be substrate dependent and may show varied results for different substrates[32]. Paraoxon is the most commonly used substrate for the determination of PON1 activity and this level of enzyme activity has been shown to reflect the antioxidant capacity of the enzyme[33]. At the baseline, PON1 activity towards paraoxon was unaffected by the differences in sex but PON1 activity towards phenyl acetate was significantly higher in male than females ( $P<0.05$ ). After four months of simvastatin treatment, PON1 activity towards phenyl acetate significantly increased in male subjects ( $P<0.05$ ), but did not changed significantly in female subjects ( $P>0.05$ ). It is rather difficult to explain this finding about mechanism underlying the differences, since the number of participants

was less to make a meaningful comparison, which is a drawback of the study. Moreover, most of participants were menopause female and more clinical evidence is required to support the effect of simvastatin on sex differences. The significant increase in PON1 activity in the current study may be due to increased PON1 concentration. Recently, Deakin and colleague had demonstrated that simvastatin increased the nuclear sterol regulating element binding protein-2. This protein binds to the paraoxonase promoter and cause enhanced promoter activity[34]. After four months of simvastatin treatment, the current study observed percent change in PON1 activity towards paraoxon was positively correlated with percent change of TAS ( $R^2=0.304$ ), which indicates PON1 reduced the extent of peroxidation and the risk of heart diseases. It is suggested that PON1 inhibits lipid peroxidation by hydrolyzing lipid peroxide and hydrogen peroxide. On the other hand, it possibly reduces oxidative stress and cause a reduction in the inactivation of PON1 and thereby leading to the increase of PON1 activity as observed after 4 months of treatment.

In the present study, simvastatin significantly reduced CD (4.4%), total peroxide (13.0%) and MDA (15.2%), whereas, TAS was significantly increased (27.3%). These parameters caused overall significant reduction of OSI (24.0%). The effect of simvastatin in lowering CD, total peroxide, MDA and increasing TAS levels obtained from our study were similar to some previously reported studies [26,30,31]. The reductions of lipid peroxide markers imply that PON functions by hydrolyzing lipid peroxides since the level of lipid peroxidation markers are decreased where as total peroxides and MDA, decreased more than CD. However, the result did not provide enough clear cut evidence to show the precise mechanism since a reduction of lipid peroxidation could partly be due to an effect from the decreased in lipid levels. In this study, the results demonstrated clearly that simvastatin caused a significant reduction of OSI in hypercholesterolaemic subjects. It seems therefore reasonable to postulate that simvastatin exerts beneficial effect on CHD, a mechanism that is beyond its lipid modifying properties. This beneficial effect may have an impact on the prevention of CHD.

In conclusion, the present study shows that simvastatin increased the TAS and PON1 activities and decreased lipid peroxidation and oxidative stress. This might be due to antioxidant or pleiotropic properties of simvastatin. A clear understanding of the effects of lipid lowering drugs on non-lipid risk factors of atherosclerosis will be helpful for selection of optimal treatment according to risk profile of individual patients. The observations from the current study can be applicable to pharmacogenetics and also essential for the development of more effective therapy. However, strong evidence are needed to substantiate the findings of the present study.

#### Conflict of interest statement

We declare that we have no conflict of interest.



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